

AP-1/Fos-TGase2 Axis Mediates Wounding-induced *Plasmodium falciparum* Killing in *Anopheles gambiae**^[S]

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Background: Wounding renders mosquitoes resistant to human malaria parasites.

Results: Genome-wide transcriptional profiling identified 53 wound response genes, including two transglutaminases. Functional studies revealed the role of AP-1/TGase2 axis in the mosquito resistance to *Plasmodium falciparum*.

Conclusion: AP-1/TGase2 is a new axis of the mosquito immune responses to malaria parasites.

Significance: Multiple signaling pathways contribute to mosquito resistance to malaria and represent new potential targets for vector control interventions.

Anopheline mosquitoes are the only vectors of human malaria worldwide. It is now widely accepted that mosquito immune responses play a crucial role in restricting *Plasmodium* development within the vector; therefore, further dissection of the molecular mechanisms underlying these processes should inform new vector control strategies urgently needed to roll back the disease. Here, using genome-wide transcriptional profiling, bioinformatics, and functional gene analysis, we identify a new axis of mosquito resistance to monoclonal *Plasmodium falciparum* infections that includes the AP-1 transcription factor Fos and the transglutaminase 2 (TGase2), a cross-linking enzyme with known roles in wound responses. We demonstrate that Fos regulates induction of TGase2 expression after wounding but does not affect expression of the components of the well characterized complement-like system. Silencing of Fos or of TGase2 aborts the wounding-induced mosquito killing of *P. falciparum*. These results reveal multiple signaling pathways that are required for efficient *Plasmodium* killing in *Anopheles gambiae*.

Malaria is the most widespread human infectious disease transmitted by an insect vector. It causes high levels of morbidity and mortality in Africa, especially in young children and

pregnant women (1). In the current absence of efficient vaccination programs, strategies for malaria elimination should be multifaceted and include drug therapy as well as mosquito vector population control (2, 3). The major malaria vectors in sub-Saharan Africa are mosquitoes of the *Anopheles gambiae* complex. The mosquito mounts a powerful immune response against *Plasmodium* infection, which kills most parasites during the first days of invasion (4). Boosting the mosquito immune system is a promising approach for blocking *Plasmodium* development within the vector to control malaria transmission (5, 6).

Invertebrates rely exclusively on the innate type of immune responses for protection against microbial infections (7, 8). These responses are controlled by signaling pathways that are remarkably well conserved across the animal phyla. Genetic analysis in *Drosophila melanogaster* revealed four major immune pathways, Toll, Imd, JAK/STAT, and JNK, that contribute to induction of effector molecules, such as antimicrobial peptides and stress response genes (8). In *A. gambiae*, homologues of the Toll and Imd pathways selectively induce target gene expression through the NF- κ B transcription factors Rel1 and Rel2, respectively (9–11). Activation of both the Rel1 and Rel2 pathways results in an efficient immune response against malaria parasites. This response is largely mediated by the mosquito complement-like system that, among others, comprises thioester-containing protein 1 (TEP1) and two leucine-rich repeat proteins, LRIM1 and APL1 (6, 11). Activation of the Rel2 pathway appears to be more efficient at blocking the development of human *Plasmodium falciparum* parasites, whereas activation of the Rel1 impacts the development of the murine parasites *Plasmodium berghei* and *Plasmodium yoelii*, often used as models to study *Plasmodium*-*Anopheles* interactions (5, 11).

Previously, we demonstrated that wounding of the mosquito by injection of water or dsRNA promotes TEP1-mediated killing of *P. falciparum* (12). The efficiency of this response was

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^[S] This article contains supplemental Tables S1–S3 and Figs. S1–S7.

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affected by the parasite genetic clonality because it was predominantly operating in infections with monoclonal, and to a lesser extent with biclonal, parasite isolates but not in infections containing three or more parasite clones. Wound response is a complex process initiated by an injury of epidermis. The hallmarks of this process are coagulation and clot formation that seal the wound site, thereby preventing hemolymph loss and limiting dissemination of infectious agents. In *Drosophila*, wound healing is similar to well characterized developmental processes that involve epithelial rearrangements, such as dorsal closure during embryonic development (13). These processes involve massive cellular movements that help to establish or to renew cell-cell contacts and are regulated by the JNK pathway (13). Regardless of the trigger, be it developmental reorganization or injury, wound healing elicits an immune response and primes the organism for what is likely to be an increased risk of infection (14).

In invertebrates, coagulation plays essential roles in morphogenesis, wound healing, and immunity and is carried out by transglutaminases (TGases),⁴ enzymes with protein cross-linking activity. TGases catalyze formation of isopeptide bonds between a free ϵ -amino group and glutamine residue in a Ca^{2+} -dependent manner (15–17) and are essential for a variety of biological processes. For instance, in the nematode *Onchocerca volvulus*, TGase-catalyzed cross-linking is crucial for the molting of third instar larvae (18), whereas in *Drosophila*, TGase1 plays an essential role in pupal morphogenesis and cuticle sclerotization brought about by cross-linking of two cuticular chitin-binding proteins, larval serum protein 2 and a putative C-type lectin (19). During immune responses in crayfish, hemolymph coagulation is achieved by TGase-mediated cross-linking of specific clotting proteins (20–22). In the horseshoe crab *Tachypleus tridentatus*, a proteolytic coagulation cascade activated by septic injury converts coagulogen into insoluble coagulin. Further cross-linking of the coagulin polymers by TGases traps and immobilizes invading pathogens at sites of injury (23, 24). Recently, TGase function in immunity was revealed in *Drosophila*, where *TGase1* mutant larvae displayed high susceptibility to infections with entomopathogenic nematodes harboring the symbiotic bacteria *Photorhabdus bacteriophora* (25).

In contrast to other insect species that rely on a single TGase, the genome of *A. gambiae* contains three genes coding for TGases. Phylogenetic analysis of TGases in *A. gambiae*, *D. melanogaster*, *Culex pipiens*, and *Aedes aegypti* revealed that AGAP009100 is an orthologue of *TGase1* conserved in all insects, whereas AGAP009098 is an orthologue of *TGase2* identified in mosquitoes of the genera *Anopheles*, *Aedes*, and *Culex* but not in *Drosophila* (26). The third TGase is encoded by AGAP009099 and is specific to *A. gambiae*, having no homologues in other species. It is expressed in the male accessory glands and cross-links proteins of the male seminal fluid into a coagulated proteinaceous plug transferred to females during mating (26).

Here, we set out to identify mosquito factors responsible for the wounding-induced *P. falciparum* killing. A genome-wide transcriptional analysis identified 53 genes whose expression was significantly regulated upon wounding, including *TGase1* and *TGase2*. We demonstrated that depletion of TGase2, but not of TGase1, restored the numbers of developing parasites to those observed in non-injected controls. Using bioinformatics and functional gene analysis by RNAi silencing, we further demonstrated that expression of *TGase2* after wounding is regulated by the AP-1 transcriptional factor Fos, the transactivator of the JNK pathway in other animals. Our study identifies the novel role of TGase2 in the elimination of *P. falciparum* and unravels the importance of the AP-1/Fos-TGase2 axis in immune responses of mosquitoes to human malaria parasites.

EXPERIMENTAL PROCEDURES

Mosquito Colony—The *A. gambiae* sensu stricto Ngousso strain was originally established in 2006 at the Institut de Recherche de Yaoundé, Cameroon (OCEAC) from larvae collected in Yaoundé. The colony belongs to the M molecular and Forest chromosomal forms (standard chromosomal arrangements). Mosquitoes were reared in the insectary at $28.0 \pm 2^\circ\text{C}$ and $80.0 \pm 5\%$ humidity with a 12/12-h dark/light cycle. Adults were fed on a 6% sugar solution through cotton pads, and the larvae diet consisted of ground fish food (Tetra).

dsRNA Production and Silencing—Fos and *TGase2* were PCR-amplified from genomic DNA with the following primers: Fos, AG356 (5'-GAGCCTCCCGATCTACTTTTGCTAC-3') and AG357 (5'-TGGTTCGGTTTAAATTTTGGCAGT-3'); *TGase2*, AG1197 (5'-GGGGAATTCGGCCGAACCTAACGATTGAAA-3') and AG1198 (5'-GGGGCGGCCGCCCGTTAGGCCATCTCATTA-3'). Amplicons were cloned into pGEM-T Easy Vector (Promega) and subcloned as a 384-bp-long EcoRI-XhoI and 356-bp EcoRI-NotI into pLL10, resulting in pLL323 and pLL556, respectively. Plasmids pLL17 (*dsTEP1*), pLL100 (*dsLacZ*), and *dsAGAP009100* (kind gift of Dr. D. Rogers, Imperial College London) were used, and the synthesis of dsRNAs was performed as described previously (27).

Gene silencing was achieved by injecting 0.2 μg of dsRNA into the thorax of 1-day-old females using a glass capillary mounted onto a Nanoject II injector (Drummond). A dsRNA targeting a bacterial gene absent from the mosquito genome (*dsLacZ*) served as a negative control in dsRNA injections. During injection, mosquitoes were immobilized by CO_2 , and non-injected mosquitoes were also exposed to CO_2 treatment.

Transcriptional Profiling Experiments and Data Analysis—To analyze changes in the transcriptome of mosquitoes after *dsLacZ* injection, we used a microarray approach. At 3 h after injection, 30 mosquitoes were frozen in liquid nitrogen and kept at -80°C . Two independent experiments were performed using unrelated mosquito populations. Total RNA was extracted using the RNeasy extraction kit (Qiagen) according to the supplier's instructions. RNA was resuspended in water to a final concentration of 0.2 $\mu\text{g}/\mu\text{L}$, and 10 μg were used for each microarray. The quality and amount of RNA were verified by measuring the optical density (OD) with Nanodrop and by an Affymetrix bioanalyzer. The complementary RNA and hybridization of the GeneChip array (GeneChip® *Plasmodium*/

⁴ The abbreviations used are: TGase, transglutaminase; GO, gene ontology; qRT-PCR, quantitative RT-PCR; CLIP, clip domain serine protease; COI, complexity of infection.

Anopheles Genome Array, Affymetrix) were performed at the IGBMC platform (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) according to Affymetrix protocols. The GeneChip arrays, designed in 2002, included probe sets for ~14,900 *A. gambiae* transcripts, which may not fully cover the mosquito genome due to incomplete genome annotation. Raw data were deposited in the Array Express archive and are available under the E-MEXP-3677 code.

Intensity data for each probe set were calculated from images generated with the GeneChip scanner and were analyzed with Bioconductor software, which implements packages from the R language. Intensities were normalized according to the robust multiarray analysis method (28) using the Affy package (29). GeneChip array data were quality-controlled using established bioinformatic methods (30) using the Affycore tools package (31). Probe sets with an absolute fold change greater than 2 were filtered, and significantly regulated probe sets ($p < 0.05$) were identified by conducting a moderated t test, which enhances statistical power compared with the classical t test by borrowing variance information from independent probe sets,⁵ followed by p value adjustment according to the method of Westfall and Young (33) using the Multtest package (34). Results were visualized in TreeView software after running a hierarchical clustering analysis with Cluster version 3.0 software (35).

To highlight gene ontology (GO) terms that were significantly enriched among the genes regulated by *dsLacZ* injection, we conducted a GO enrichment test using the DAVID Bioinformatics Resources 6.7 Web site.

To identify genes potentially regulated by the same pathway, we conducted a k -means clustering analysis that groups genes with respect to similarity of their expression profiles. Affymetrix probe set expression data for the genes significantly regulated after *dsLacZ* injection and for *Fos* and *Jun* were retrieved from the *A. gambiae* Gene Expression Database at the University of California Irvine for the following conditions: BF3 (3 h post-blood feeding), BF24 (24 h post-blood feeding), and NBF (non-fed). The retrieved microarray data were normalized together with our microarray data as described above. k -means clustering analysis was conducted with $k = 4$ using the Stats package (36).

Quantification of Gene Expression by Quantitative RT-PCR (qRT-PCR)—To validate the results obtained with the GeneChip arrays, we compared the expression levels of 10 genes between microarray and qRT-PCR. Total RNA from 10 mosquitoes (non-injected control and *dsLacZ*-injected) was extracted 3 h after injection using TRI Reagent® (Molecular Research Centre, Inc.). Three independent biological replicates from different mosquito generations were performed. Reverse transcription was achieved using the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Gene transcript levels were quantified using Fast SYBR Green® master mix (ABI) in an ABI 7500 Fast Real-time PCR machine. Primers are detailed in [supplemental Table S2](#). The housekeeping gene *RPL19* was used to

normalize transcript levels, and expression levels in *dsLacZ*-injected mosquitoes were compared with non-injected controls. To determine a correlation between results obtained by qRT-PCR and by microarray, we conducted a linear regression analysis. The p value reported for the regression equation represents the result of the test that estimates the null hypothesis of the slope as being equal to zero, whereas the p value reported after the coefficient of correlation tests for the significance of the correlation coefficient.

To quantify the transcript levels of *AGAP009100* (*TGase1*), *AGAP009098* (*TGase2*), *TEP1*, *APL1C*, *LRIM1*, and *CTL4* ([supplemental Table S2](#)) at different time points (1.5, 3, 4.5, and 6 h) after *dsLacZ* and *dsFos* injection, we used qRT-PCR. Three independent biological experiments were conducted. Fold change after injection of *dsLacZ* and *dsFos* was calculated relative to non-injected controls. A two-way analysis of covariance was applied to determine the statistical significance of the effects of dsRNA treatment and time on transcript abundance. The t test was used to identify significant differences in expression levels between control (*dsLacZ*) and *dsFos*-injected mosquitoes for each time point separately.

To verify the efficiency of *TEP1*, *TGase1*, *TGase2*, and *Fos* knockdown, we used qRT-PCR as described above. RNA was extracted from 10 mosquitoes 4 days after injection of dsRNA, reverse-transcribed, and amplified using primers described in [supplemental Table S2](#). Three independent experiments were performed, and expression levels were normalized to *RPL19* expression.

Immunoblotting—Hemolymph of 15 dsRNA-treated females was collected 3–4 days after injection into a Laemmli protein-loading buffer by proboscis clipping. Hemolymph samples were separated on 8% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked by incubation in a 5% milk powder solution for 1 h; rinsed in PBS, 1% Triton X-100; and incubated either with rabbit anti-TEP1 (1:500) or with anti-prophenoloxidase (1:15,000) antibody solutions for 1 h. After PBS washes, the membrane was incubated in a 5% milk powder PBS solution containing a secondary anti-rabbit antibody (1:15,000) tagged with horseradish peroxidase (HRP). The enhanced chemiluminescence kit (Amersham Biosciences) was used to reveal the blot.

Experimental Infections with *P. falciparum* Gametocytes from Blood Donors—Mosquito infections were carried out in 2007 and in 2010 at OCEAC with the *P. falciparum* gametocytes collected from the blood of children (5–11 years old) who attended schools in the area of Mfou (3°40' north; 11°35' east), a small city 30 km from Yaoundé. Parasites were detected by Giemsa-stained thick blood smears. Children showing asexual parasitemia were treated with artemisinin-based combination therapy according to national guidelines. Asymptomatic gametocyte-positive children were enrolled as volunteers upon signing of an informed consent form by their legal guardian. The recruitment procedures and experimental protocols were approved by the Cameroonian ethical review committee.

For each volunteer, 4 ml of venous blood were collected by venipuncture in heparinized Vacutainer tubes and centrifuged at $2000 \times g$ for 5 min at 37 °C. The supernatant, containing

⁵ D. M. Witten and R. Tibshirani, unpublished data.

serum, was replaced by non-immune AB serum to limit transmission-interfering factors (37). The mixture (350 μ l) was dispensed into prewarmed glass feeders, on which mosquitoes previously starved for 12 h were allowed to feed for 30 min through a parafilm membrane. Well-fed mosquitoes were maintained in the insectary at 28 °C. After 7 days, midguts were dissected, stained with a 0.4% mercurochrome solution, and examined under light microscopy ($\times 200$) for oocyst counts. Significance of differences in oocyst numbers (only for samples with at least one oocyst per midgut) and in prevalence of infection was assessed by computing the standardized mean differences and the odds ratio, respectively, under random effect models using comprehensive meta-analysis software (38).

Genotyping of *P. falciparum* Gametocytes—Gametocyte filtration and DNA extraction for the samples collected in 2010 were performed as described (12). Purified gametocytes were genotyped at seven microsatellite loci (39): POLY α (chromosome 4, GenBankTM ID G37809), TA87 (chromosome 6, G38838), TA109 (chromosome 6, G38842), ARA2 (chromosome 11, G37848), Pfg377 (chromosome 12, G37851), PfPK2 (chromosome 12, G37852), and TA60 (chromosome 13, G38876). Amplification procedures were performed as described previously (40). The fluorescent PCR products were examined on an AB Prism 3100 Genetic Analyzer (Applied Biosystems) relative to Lys-500 as an internal size standard and examined with GeneMapper software (Applied Biosystems).

RESULTS AND DISCUSSION

Identification of Wound Response Genes by Genome-wide Expression Analysis—We posited that wounding-induced mosquito resistance to human malaria parasites resulted from a transcriptional up-regulation of wound response genes prior to *P. falciparum* infection. To identify these genes, the transcriptome of *A. gambiae* females 3 h after injection of dsRNA (*dsLacZ*) was compared by DNA-based Affymetrix GeneChip arrays with the transcriptome of control age-matched untreated females from sibling cohorts. The choice of this time point was based on our previous observations on *TEP1*, whose expression was transiently induced by wounding shortly after injection (6). Injections of *dsLacZ* served as a proxy for wounding and allowed a direct comparison of parasite infection phenotypes with our earlier study (12). Among 141 genes whose expression was modulated (induced or repressed) by injection more than 2-fold, 53 genes displayed statistically significant ($p < 0.05$) regulation; seven genes were down-regulated (13.2%), whereas 46 were up-regulated (86.8%) (supplemental Fig. S1A and Table S1). Expression profiles of 10 of 53 genes were gauged by qRT-PCR (supplemental Table S2) and compared with the microarray data. Linear regression between the two data sets yielded a coefficient of determination ($y = 0.84x + 0.51$; $P_{(1,8)} = 0.006$) and a slope of regression ($R^2 = 0.64$; $p = 0.027$) close to 1, demonstrating a good correlation between the results obtained by microarrays and by qRT-PCR, thereby validating the values obtained by the microarray approach (supplemental Fig. S2 and Table S3). A previous study identified 81 genes, whose expression was regulated at 1, 6, 12, or 24 h after wounding (41). Only one wound response gene, *FBN9* (AGAP011197), was identified by both studies. Methodological

differences may explain these results. The previous study was performed with the first generation of cDNA microarrays, representing around 3840 expressed sequence tag clones, and measured transcript abundance at the time points not overlapping with our study. We showed that wounding induces transient changes in gene expression and therefore is sensitive to the timing of the analysis. Additional minor differences may have contributed to the observed discrepancy; the previous study used a different mosquito line (L3-5), wounding consisted of pricking mosquitoes with a needle, and the microarray design was based on expressed sequence tags cloned from a hemocyte-like cell line. Last, distinct statistical methods were used for data analysis.

GO analysis revealed a high proportion of wound response genes that encoded proteins with a potential role in proteolysis-related processes (24.5%). For comparison, the second largest GO class comprised the genes encoding proteins potentially involved in signal transduction, which represented 3.7% of all regulated genes (supplemental Fig. S1B). In line with these data, proteolysis represented the only biological process significantly ($p < 0.001$) regulated in our data set, and proteolysis-related genes were enriched 5-fold compared with their proportion in the annotated *A. gambiae* genome. Proteolysis is known to be involved in responses to injury in a number of organisms, where it swiftly activates such processes as blood coagulation, fibrinogenesis, clotting, and angiogenesis (22). Among proteolysis-related genes, we identified 11 clip domain serine proteases (CLIPs), enzymes that trigger extracellular proteolytic cascades in invertebrates (Fig. 1A and supplemental Table S1). Three CLIPs have known functions in mosquito immune responses and/or in interactions with *P. berghei*. *CLIPB15* has been shown to promote parasite killing by lysis (42), whereas *CLIPA2* and *CLIPA8* are required for ookinete melanization (43, 44). Our analysis identified several non-CLIP serine proteases. One of them, immune-induced serine protease 1 (*ISRP1*, AGAP005194), was previously reported to be induced by LPS injection (45). In these experiments, LPS-injected mosquitoes were compared with non-injected controls. Therefore, it is possible that expression of this protease is induced by injection only. Moreover, *ISRP1* was identified in a transcriptional profiling of genes regulated by mating in the female reproductive organs (46). Expression of *ISRP1* was shut down in the female atrium at 24 h postmating. The exact roles of this protease in immunity and reproduction remain to be elucidated. The activity of serine proteases is tightly controlled by serine protease inhibitors (serpins). We detected one wound response gene (AGAP00137) coding for serpin 11 (SRPN11), previously reported to be expressed throughout the mosquito life cycle (47).

Several other genes with demonstrated functions in anti-*Plasmodium* responses were identified. Interestingly, most of them are regulated by the members of the NF- κ B family, Rel1 and/or Rel2 (11, 48). Fibrinogen domain immunoglobulins (FBN) are common pattern recognition receptors in invertebrates. Two FBN-encoding genes were up-regulated upon wounding, including *FBN9*, a known antiplasmodium factor (49). Expression of three *TEP* genes (*TEP1*, *TEP3*, and *TEP12*) was significantly up-regulated by wounding. *TEP1* and *TEP3* cooperate to

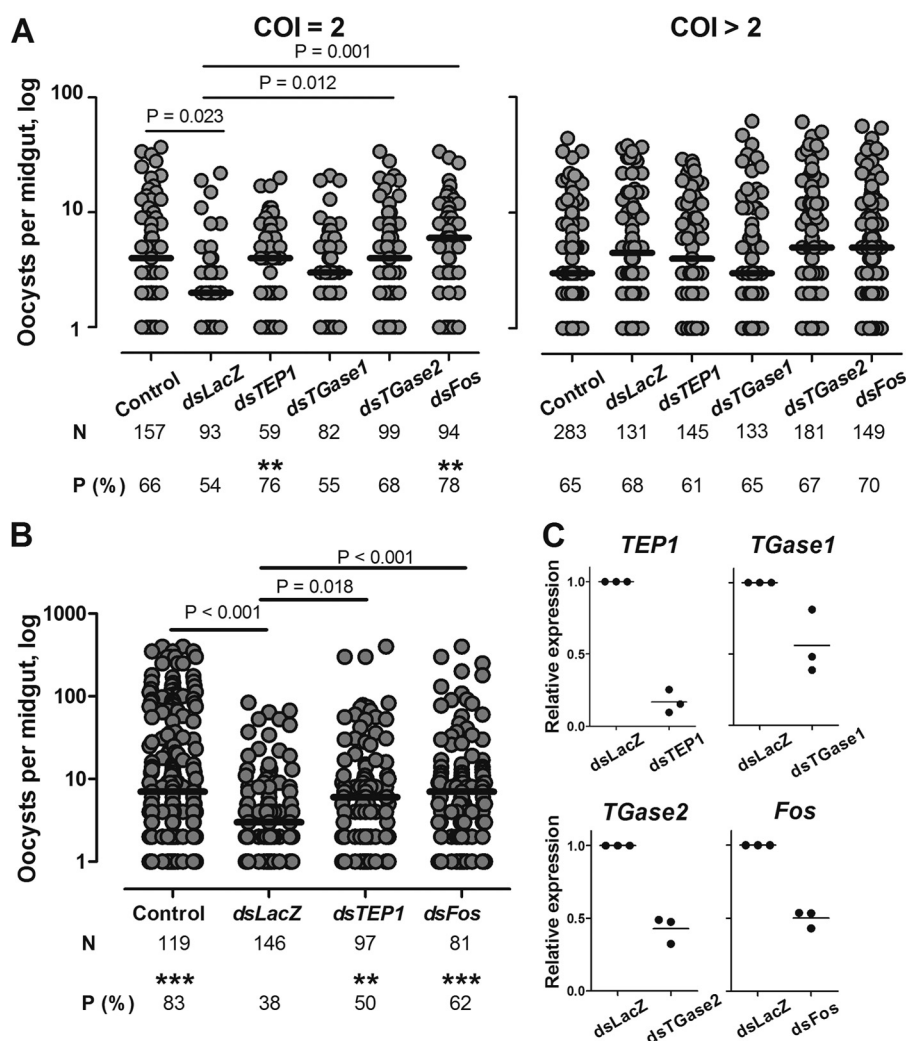


FIGURE 1. Effect of *TGase1*, *TGase2*, and *Fos* silencing on *P. falciparum* development in the mosquito. Mosquitoes were injected with dsRNA against *TEP1*, *TGase1*, *TGase2*, and *Fos* (A) or *TEP1* and *Fos* (B), respectively. Non-injected (Control) and dsLacZ-injected mosquitoes were used as controls. After injection, mosquitoes were infected with four biclonal (COI = 2) and six polyclonal (COI > 2) *P. falciparum* isolates in A or with four other isolates in B. Mosquito midguts were dissected 7 days after infection. Only samples with at least one oocyst are plotted, and they represent the infection intensity. Horizontal bars indicate medians. N, total numbers of mosquitoes dissected; P (%), percentage of infected mosquitoes (prevalence). Significant differences in prevalence are indicated by two asterisks ($p < 0.01$) and three asterisks ($p < 0.001$) above the prevalence values. C, knockdown efficiency was examined by quantitative RT-PCR performed in three independent experiments (black dots) for *TEP1*, *TGase1*, *TGase2*, and *Fos*. Expression levels of each gene after dsRNA injection were compared with the levels in dsLacZ-injected control mosquitoes. Horizontal bars represent means.

promote phagocytosis of Gram-positive and Gram-negative bacteria (50) and in antiparasitic responses (51, 52). TEP1 circulates in the mosquito hemolymph as a trimeric complex comprising two leucine-rich repeat proteins, APL1C and LRIM1 (53, 54). Levels of APL1C and LRIM1 transcripts were also increased by wounding, confirming our previous observation that the mosquito complement-like system is involved in the wound responses (12). Wounding also induced the transcript abundance of another leucine-rich repeat-encoding gene, APL2 (or LRRD7), which was reported to affect both *P. berghei* and *P. falciparum* development, and of two genes encoding C-type lectins, CTLMA2 and CTL4, whose depletion caused melanization of *P. berghei* ookinetes in the mosquito midguts (55–57).

A subset of the wound response genes encoded diverse proteins with potential functions in tissue repair or cuticle formation. Abundance of AGAP001508 transcripts, a *Drosophila* homologue of a gene encoding a structural protein of the insect cuticle was 6-fold induced by wounding (supplemental Table S1) (58, 59).

Wound response genes also included AGAP003720, a homologue of the *Drosophila* annexin X (supplemental Table S1), which has been implicated in wounding-induced membrane resealing in *Drosophila*, and AGAP009145, a homologue of a *Drosophila* protein involved in DNA repair. In contrast, transcript levels of *flightin* (AGAP007249), whose homologue in adult *Drosophila* regulates somatic development of the flight muscles (60–62), were repressed. The gene encoding ICHIT (AGAP006434) contains two putative chitin-binding domains. Although its expression has been reported to be induced by *Plasmodium* and bacteria infections (63), the ICHIT transcript level was down-regulated by wounding as well as the cuticle protein family-like CPFL6 (AGAP010907). Finally, transcript levels of two genes, AGAP008963 and AGAP012399, that encode α -amylases, were also down-regulated by wounding. These genes are massively expressed in the midgut of males and females and might be involved in glucose degradation during sugar feeding (64).

TGase2-mediated Killing of *P. falciparum* in *A. gambiae*

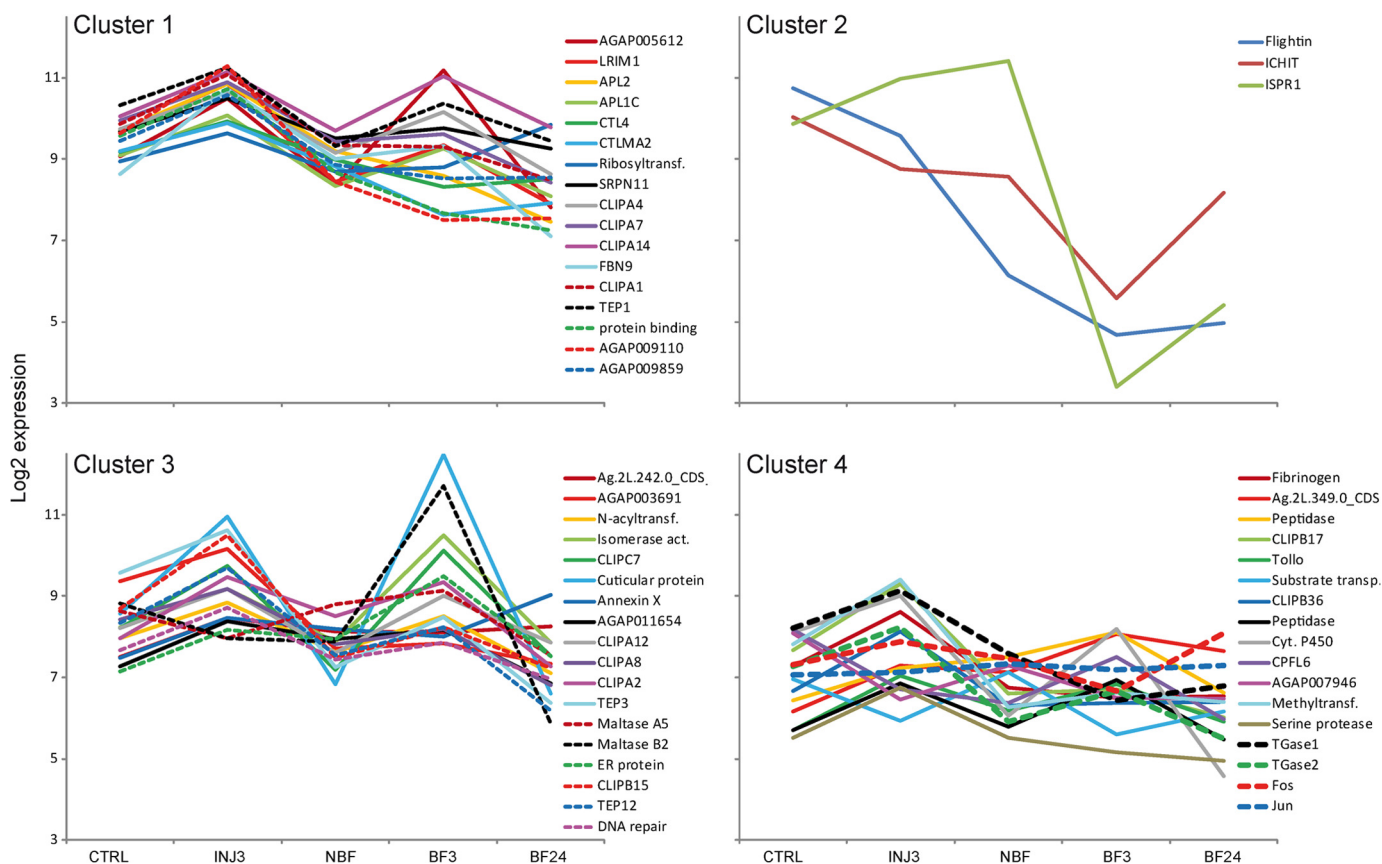


FIGURE 2. **k-means clustering analysis of the expressional profiles of the genes regulated by wounding.** Clusters represent genes with similar expressional profiles. Transcript abundance was quantified using microarray data for the following conditions: non-injection, injection (3 h after *dsLacZ* injection), BF3 (3 h after blood feeding), BF24 (24 h after blood feeding), and NBF (nonfed). Data for the last three conditions were retrieved from the *A. gambiae* Gene Expression Database at the University of California Irvine.

TGase2 Contributes to the Mosquito Immune Responses against *P. falciparum*—Among wound response genes, we detected two *TGase*-encoding genes, *AGAP009100* (*TGase1*) and *AGAP009098* (*TGase2*). To test whether these *TGases* contributed to mosquito resistance to *P. falciparum* after wounding, we silenced expression of the corresponding genes by injecting *dsRNA* into adult females. In these experiments, we used three control groups: (i) non-injected mosquitoes from the same cage as a control for infection levels; (ii) *dsLacZ*-injected mosquitoes as a control for wounding-induced parasite killing; and (iii) *dsTEP1*-injected mosquitoes as a positive control (12). Silencing efficiency was validated using qRT-PCR (Fig. 1C). Injected and control mosquitoes were fed 3–4 days after *dsRNA* injection with the blood of 10 *P. falciparum* gametocyte carriers. Substitution of donor sera with non-immune sera was performed to eliminate possible effects of donor blood composition and of immune factors on parasite development. Because the genetic complexity of gametocytes impacts the levels of *P. falciparum* infections and the efficiency of parasite killing by the mosquito immune response, we purified and genotyped gametocytes in the donor blood (12). All 10 gametocyte carriers contained 2–4 *P. falciparum* clones (supplemental Table S4). Results of experimental infections were analyzed separately for two groups according to the complexity of infection (COI) of the *P. falciparum* isolates: (i) four biclonal isolates (COI = 2) and (ii) six polyclonal isolates (COI > 2).

Consistent with our previous results (12), the overall oocyst levels in polyclonal infections were low. In the experiments with biclonal parasites, the infection intensity in non-injected mosquitoes (Control) was significantly higher than in *dsLacZ*-injected mosquitoes (Fig. 1A and supplemental Table S4). Silencing *TGase2* restored the levels of infection intensity to the levels in non-injected mosquitoes, which were significantly higher than after wounding. In contrast, in mosquitoes depleted for *TGase1*, oocyst numbers per midgut were similar to the ones in *dsLacZ*-injected mosquitoes. Silencing of *TEP1* resulted in a significant increase of the prevalence of infection when compared with *dsLacZ* mosquitoes and a non-significant increase in infection intensity. Medians of infection intensity were similar between *dsTGase2*- and *dsTEP1*-injected mosquitoes. These results suggest that *TGase2* mediates wounding-induced killing of malaria parasites. Consistent with our previous study (12), no effect of wounding or of gene silencing was observed in polyclonal infections (COI > 2) (Fig. 1A).

Levels of *TGase2* transcripts increased within the first 3 h after *dsLacZ*-injection. How does this transient *TGase2* up-regulation impacts *P. falciparum* development 3–4 days later? We believe that wounding, similar to Cactus depletion, boosts the efficiency of parasite killing (6). However, better understanding of the *TGase2* role, direct or indirect, in parasite killing will be required to answer this question.

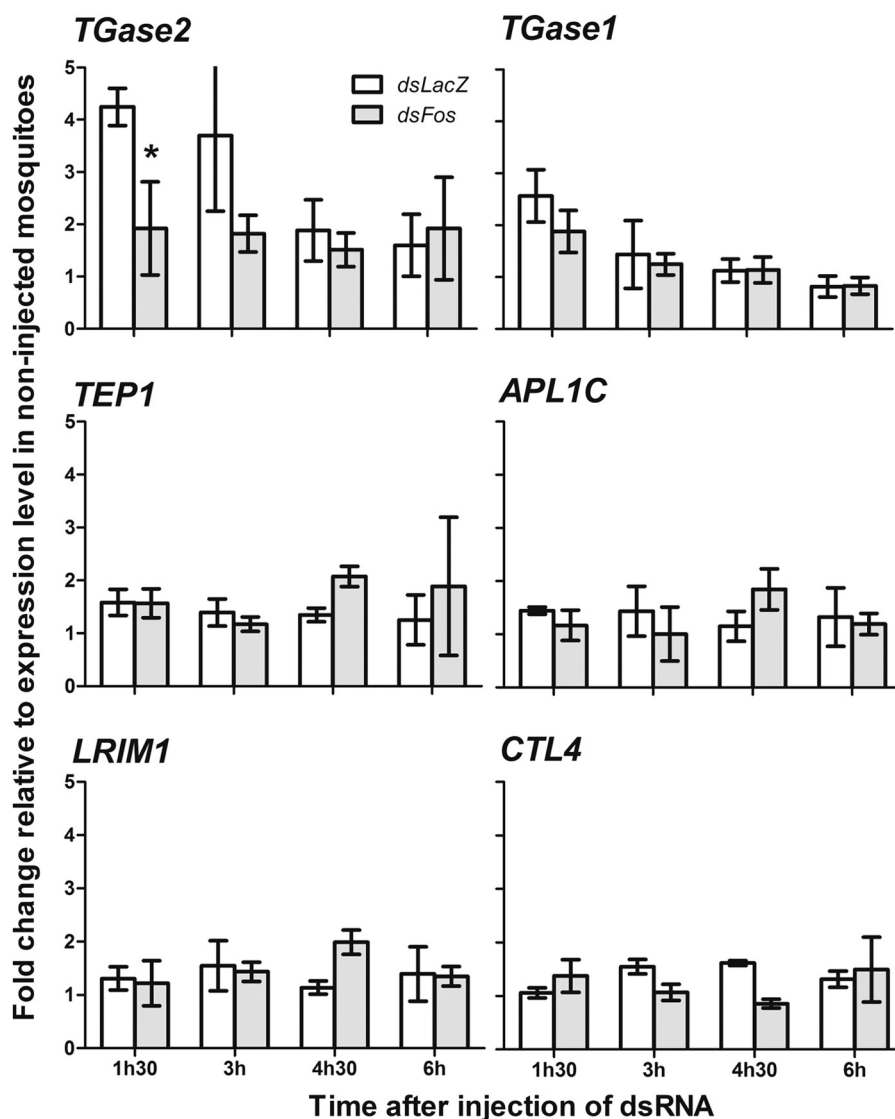


FIGURE 3. **Fos-mediated regulation of *TGase2*, *TGase1*, *TEPI*, *APL1*, *LRIM1*, and *CTL4* expression after wounding.**—Fold change \pm S.E. (error bars) relative to non-injected mosquitoes was calculated for *TGase1*, *TGase2*, *TEPI*, *LRIM1*, *APL1C*, and *CTL4* genes at 1.5, 3, 4.5, and 6 h after injection of *dsLacZ* and *dsFos* using qRT-PCR. Non-injected mosquitoes of the corresponding time were used as controls. The *RPL19* housekeeping gene was used for normalization. *, significant difference (t test, $p < 0.05$) in transcript abundance between *dsLacZ*- and *dsFos*-injected mosquitoes.

Fos Regulates *TGase 2* Expression after Wounding—We next sought to identify the signaling pathway(s) that regulate wounding-induced expression of *TGase2*. Results of our microarray analysis failed to reveal any components of signaling pathways whose expression was induced by wounding (supplemental Table S1). Therefore, we examined the promoter region of *TGase2* for transcription factor binding motifs using Tf site scan software (65). A number of potential transcription factor binding sites were identified, including two binding sites for activator protein 1 (AP-1) (TGAGTCA) in the 1-kb region upstream of the TATA box. In *Drosophila*, AP-1 transcription factors function as heterodimers of Jun and Fos proteins that bind to AP-1 DNA motif and mediate activation of the JNK pathway (66), one of the major regulators of wounding responses in *Drosophila* (67). Because we did not detect any evidence of *Jun* or *Fos* up-regulation 3 h after wounding in the microarray data, we increased the resolution power of our analysis by including additional experimental data retrieved from

the University of California Irvine *A. gambiae* Gene Expression Profile Web site. We then grouped the wounding-induced genes identified here and *Fos* (AGAP001093) and *Jun* (AGAP006386) according to their expressional profiles in control, *dsLacZ*-injected mosquitoes (3 h postinjection) and before and 3 and 24 h after blood feeding in non-injected mosquitoes by applying a *k*-means clustering analysis (Fig. 2 and supplemental Table S5). Co-regulated genes often belong to the same signaling pathway. Our analysis identified three clusters containing 14–16 genes and one small cluster of three genes. Cluster 1 encompassed the most potent antiparasitic genes, such as *TEPI*, *FBN9*, *LRIM1*, and *APL1C*, two genes with a reported protective effect on *Plasmodium* (*CTL4* and *CTLMA2*), and four *CLIPA* genes (*CLIPA1*, -4, -7, and -14). In line with these results, *TEPI*, *LRIM1*, *APL1C*, and *CTL4* are co-regulated through the Rel1/Cactus and Rel2 pathways (6, 48). Given that co-regulated genes often display similar functions, other genes of the cluster may also have important roles

in responses of mosquitoes to malaria parasites. Cluster 2 grouped three genes: *ISPR1*, *Flightin*, and *ICHIT*. Cluster 3 included genes with diverse functions: *TEP3* and *-12*, three genes with potential roles in tissue repair, genes coding for proteolysis-related proteins, such as five *CLIP* genes (*CLIPA2*, *-A8*, *-A12*, *-B15*, and *-C7*), and the two α -amylases repressed by wounding. Cluster 4 contained *Tollo*, *CPFL6*, *fibrinogen* (*AGAP005848*), and two proteolysis-related *CLIP* genes (*CLIPB17* and *-36*) but most importantly, *TGase1*, *TGase2*, *Fos*, and *Jun*, suggesting that these genes may be part of the same signaling pathway.

To examine whether expression of the *TGases* after wounding was indeed regulated by AP-1, we compared expression levels of *TGase1*, *TGase2*, *TEP1*, *APL1C*, *LRIM1*, and *CTL4* at different time points after injection of *dsLacZ* and *dsFos*. Changes in the transcript abundance at the indicated time points after injections were calculated relative to non-injected control mosquitoes. Injection of *dsFos* significantly decreased levels of *TGase2* transcripts after wounding (two-way analysis of covariance, RNAi effect: $F_{(1,20)} = 6.83$; $p = 0.017$) but had no effect on transcript abundance of other tested genes (Fig. 3 and supplemental Table S6). Immunoblotting analysis further confirmed at the protein level that silencing of *Fos* did not affect *TEP1* expression at 24 and 48 h after wounding (supplemental Fig. S3). Interestingly, depletion of *Fos* had no effect on transcript levels of *TGase1*, although the latter clustered with *Fos* in the *k*-means analysis (Fig. 2 and supplemental Table S5) and contained three potential AP-1 binding motifs in its promoter region. Because our analysis also identified one potential NF- κ B binding site in its promoter region, AP-1 and Rel1/Cactus modules may cooperate in regulating *TGase1* expression after wounding.

Fos* Contributes to the Mosquito Resistance to *P. falciparum—To further ascertain the role of *Fos* in the wounding-induced *P. falciparum* killing, we compared infection levels in *dsLacZ*- and *dsFos*-injected mosquitoes using donor blood infected with field isolates of *P. falciparum*. Gene silencing was conducted on the 10 polyclonal isolates previously used and on four additional isolates collected earlier in the spring of 2007, for which genotyping was not performed. Several characteristics of these infections (e.g. high infection rates and sensitivity to injection and to *TEP1* depletion) were reminiscent of monoclonal infections (12) (Fig. 1B and supplemental Table S7). In both sets of experiments, we observed a significant increase in infection intensity and in oocyst prevalence in *dsFos*-injected mosquitoes as compared with *dsLacZ* controls for biclonal isolates and for infections carried out in 2007 (Fig. 1, A and B, respectively). Taken together, our results strongly suggest that AP-1/*Fos* contributes to the wounding-induced killing of *P. falciparum* through direct or indirect regulation of *TGase2* expression and that *Fos*/*TGase2* constitute a novel axis in the antiparasitic responses of *A. gambiae*.

CONCLUSIONS

Here we report that AP-1/*Fos*-regulated transglutaminase 2 restricts development of human malaria parasite in *A. gambiae*. In *Drosophila*, *TGase1* has been previously implicated in immune defenses against bacteria (25), but this is the first

report that demonstrates antiparasitic properties of *TGases*. How *TGase2* contributes to *P. falciparum* killing is currently unknown. It is also unclear whether *TGase2* requires the function of *TEP1*, which also mediates wounding-induced parasite killing (12). Interestingly, our results suggest that distinct signaling pathways regulate wound responses; for instance, expression of *TEP1* is regulated by the Rel1/Rel2 module but not by AP-1/*Fos* (6). In contrast, induction of *TGase2* expression after wounding is controlled by AP-1/*Fos*. This is the first indication of the role of the component of the AP-1 transcriptional factor in the antiparasitic responses of mosquitoes to the human malaria parasite, *P. falciparum*. A recent study using a rough eye screen in *Drosophila* revealed a genetic interaction between the JNK pathway and *TGase1* (32). Results reported here extend this observation to mosquito immune responses. In the natural setting, mosquitoes are exposed to a rough environment that causes frequent losses of appendices and exposes mosquitoes to predator insults that could induce wound responses, thereby modulating mosquito vector competence. The identification of pathways responsible for the antiparasitic response in *A. gambiae* is not only crucial for the design of efficacious vector control strategies based on the release of transgenesis-engineered refractory mosquitoes but also illustrates the diversity of mechanisms that insects employ to cope with diverse pathogens.

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